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Creating resistance to avian influenza infection through genome editing of the ANP32 gene family.

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Key words: avian influenza, genome editing, poultry, viruses, disease resistance

Abstract

Chickens genetically resistant to avian influenza could prevent future outbreaks. In chickens, influenza A virus (IAV) relies on host protein ANP32A. We used CRISPR/Cas9 to generate homozygous gene edited (GE) chickens containing two ANP32A amino acid substitutions that prevent viral polymerase interaction. After IAV challenge, 9/10 edited chickens remained uninfected. Challenge with a higher dose, however, led to breakthrough infections. Breakthrough IAV virus contained IAV polymerase gene mutations that conferred adaptation to the edited chicken ANP32A. Unexpectedly, this virus also replicated in chicken embryos edited to remove the entire ANP32A gene and instead co-opted alternative ANP32 protein family members, chicken ANP32B and ANP32E. Additional genome editing for removal of ANP32B and ANP32E eliminated all viral growth in chicken cells. Our data illustrate a first proof of concept step to generate IAV-resistant chickens and show that multiple genetic modifications will be required to curtail viral escape.

34 **Main**

35 Influenza A viruses (IAVs) are enveloped negative-sense single stranded RNA viruses which infect birds
36 and mammals causing respiratory disease and significant economic losses[1–3]. Avian influenza in poultry
37 poses a constant zoonotic threat to humans with the possibility for evolution of novel IAVs with
38 pandemic potential[4]. At the current time, a highly pathogenic avian influenza virus H5N1 subtype clade
39 2.3.4.4b is geographically dispersed across Asia, Europe, Africa and the Americas, associated with wild
40 birds die offs, devastating impacts on farmed poultry and numerous incursions into mammals
41 including some human cases and deaths[5]. Poultry vaccination for control of avian influenza is not
42 reliable due to the rapid antigenic drift of field viruses and is controversial due to political and
43 economic implications[6].

44 The heterotrimeric viral RNA-dependent RNA polymerase, comprised of PB1, PB2 and PA proteins, is
45 responsible for transcription and replication of the IAV genome in the host cell nucleus, but depends
46 on essential support from the host-encoded ANP32 family of proteins, ANP32A and ANP32B[7]. An
47 important difference exists between avian and mammalian ANP32A, whereby the avian protein has
48 an additional 33-amino-acid sequence between its N-terminal leucine rich region (LRR) and the C-
49 terminal low complexity acidic region (LCAR) domains. The shorter mammalian ANP32 proteins do not
50 efficiently support avian influenza polymerase and this accounts for a host range restriction that limits
51 the infection of humans when exposed to infected birds[8]. However, the virus can acquire mutations,
52 usually in PB2 or PA subunits, that adapt polymerase to use mammalian ANP32 proteins, or can
53 acquire these segments from a mammalian-adapted virus by a process of reassortment, and this is a
54 prelude for the emergence of pandemic influenza[1]. In human cells, ANP32A and ANP32B serve
55 redundant roles to support influenza polymerase[9]. In chicken cells, ANP32A is solely responsible for
56 the pro-viral function while ANP32B is inactive[10]. In both species, ANP32E is suggested to have an
57 antiviral effect[11]. Replication of the viral genome requires the formation of a replicative platform
58 consisting of two heterotrimeric polymerase molecules bridged by ANP32A to form an asymmetric
59 dimer[12]. The amino acids 129N and 130D in the fifth leucine-rich repeat (LRR) of ANP32A are critical
60 for this interaction. Chicken ANP32B contains the amino acids 129I and 130N, and does not interact
61 with the viral polymerase, accounting for its inability to support polymerase activity[10,13]. Here, we
62 tested the hypothesis that IAV infection and transmission will be abrogated in chickens containing the
63 two N129I and D130N substitutions engineered into ANP32A. We used genome editing (GE) to alter
64 these residues in the ANP32A gene and generated ANP32A-GE chickens to test for resistance to avian
65 influenza infection.

66 **Results**

Generation of genome-edited primordial germ cells

To generate ANP32A-GE (ANP32A$^{N129I\text{-}D130N}$) cells and chickens, we applied CRISPR/Cas9 and a short single-stranded oligonucleotide (ssODN) template to introduce a 3-nucleotide base-pair change in exon 4 of ANP32A creating a two-amino-acid substitution **(Fig. 1a)**[14,15]. We targeted the locus in *in vitro* propagated male and female chicken primordial germ cells (PGCs) and then cultured single PGCs to establish clonal GE cell lines **(Fig. 1b; Supplementary Fig. 1**). Sanger sequencing of clonal cells identified cells containing biallelic edits. We examined the top predicted CRISPR/Cas9 off-target sites and detected no off-target mutations in selected GE clonal lines (**Supplementary Fig. 2**). We also confirmed that ANP32A protein expression levels were not altered in the GE PGC lines using western blot analysis **(Supplementary Fig. 3)**. Principal component analysis (PCA) and heat maps generated from an RNA transcriptome analysis showed that the PGCs cluster by sex and cell line rather than the ANP32A genotype of the cells, indicative of no significant changes in the transcriptome of the edited cells (**Supplementary Fig. 4-5**).

IAV polymerase activity is restricted in ANP32A$^{N129I\text{-}D130N}$ edited chicken cells

We, and others, have previously shown that exogenously expressed chicken ANP32A variants containing either the N129I or D130N amino acid substitutions fail to complement avian influenza polymerase function in human cells or in chicken cells that lack wild type ANP32A[10,13]. Here, we directly assessed IAV polymerase activity in the ANP32A$^{N129I\text{-}D130N}$ edited chicken cells. We differentiated PGC lines harbouring the ANP32A$^{N129I\text{-}D130N}$ edit, or an edit designed to abrogate ANP32A expression by a small deletion in exon 1 (ANP32A^{knockout}) or WT PGCs into fibroblast-like cells, which are permissive for IAV replication and minigenome polymerase assays[10], and assessed the activity of the reconstituted avian IAV polymerase (PB2 627E) or the human-adapted isoform (PB2 627K) from three avian influenza A viruses: H9N2-UDL virus (A/chicken/Pakistan/UDL-1/2008, a low pathogenic virus of the predominant G1 lineage), H5N1 50-92 virus (A/turkey/England/50-92/1991, representing a highly pathogenic avian influenza virus with no record of zoonosis) and H5N1 Tky05 virus (A/turkey/Turkey/1/2005, a highly pathogenic avian virus that has frequently infected humans). All IAV polymerases were active in wildtype cells but inactive in either ANP32A$^{N129I\text{-}D130N}$ or ANP32A^{knockout} cells (**Fig. 1c; Supplementary Fig. 6);** exogenous expression of wildtype chicken ANP32A rescued polymerase activity in ANP32A$^{N129I\text{-}D130N}$ cells. These results confirm that the residues at position 129 and 130 of chicken ANP32A are key determinants of IAV polymerase activity in chicken cells.

99 **Generation of ANP32A$^{N129I-D130N}$ GE chickens using sterile surrogate hosts**

100 We generated surrogate host chickens producing only gametes that harbour the ANP32A$^{N129I-D130N}$ edit
101 through microinjection of male and female GE PGCs into iCaspase9 sterile host embryos (**Fig. 1a;**
102 **Supplementary Fig. 1; Supplementary Table 1**)[16]. Subsequent mating of the mature surrogate host
103 male and female chickens (Sire Dam Surrogate mating) generated homozygous ANP32A$^{N129I-D130N}$ eggs
104 and chicks for analysis in a single generation **(Supplementary Table 2).** As ANP32A function is
105 associated with the development of bone, cartilage, brain and heart in mouse models, we monitored
106 the growth of ANP32A$^{N129I-D130N}$ chick embryos to identify any health or developmental defects[17,18]. We
107 observed no developmental abnormalities in ANP32A$^{N129I-D130N}$ embryos and proceeded to hatch a
108 small cohort of GE chicks consisting of seven ANP32A$^{N129I-D130N}$ chicks (generation G_1 comprising 4
109 females and 3 males). In this preliminary experiment, we did not observe any differences in growth,
110 external appearance, behaviour, or vaccination response in comparison to wildtype controls (**Fig. 1d;**
111 **Supplementary Fig. 7-9)**. All four ANP32A$^{N129I-D130N}$ hens began laying at 20 weeks of age and
112 subsequent egg production was comparable to wildtype hens (data not shown).

113

114 **ANP32A$^{N129I-D130N}$ chickens are resistant to low-dose IAV infection**

115 To assess the susceptibility of the GE chickens to IAV infection and transmission, 10 wildtype (WT) and
116 10 ANP32A$^{N129I-D130N}$ 2-week-old chickens were intranasally inoculated with 10^3 PFU of the low
117 pathogenic avian influenza H9N2-UDL virus in separate isolators. **(Fig. 2a)**. 24 hours post-inoculation,
118 10 naive WT sentinel birds were introduced into the WT isolator and 10 naive GE sentinel birds were
119 introduced into the ANP32A$^{N129I-D130N}$ isolator to assess onward virus transmission. 4 directly
120 inoculated birds and 4 sentinel birds were sacrificed from each isolator on day 3 post-inoculation (pi)
121 for post-mortem examination. Throughout the 14-day observation period, no clinical signs were
122 observed in any birds and post-mortem analysis revealed no pathological lesions.

123 As the H9N2-UDL virus used in the challenge replicates predominantly in the respiratory tract of
124 chickens, oropharyngeal swabs were collected from day 0 to day 7 pi to assess the shedding of
125 infectious virus by plaque assay[19]. Infectious virus was detected consistently in all directly inoculated
126 WT birds (10 of 10 birds) from day 1 pi until day 4 pi with daily mean titres above 3×10^4 PFU/ml, after
127 which titres reduced and virus was cleared by day 6 pi (**Fig. 2b**). 7 of 10 co-housed WT sentinel birds
128 acquired infection by the direct exposure route from the directly inoculated birds and shed virus from
129 day 3 to day 6 post exposure (pe) (**Fig. 2b**).

130 In contrast to the WT birds, oropharyngeal shedding of infectious virus was not detected in 9 of 10
131 directly inoculated ANP32A$^{N129I-D130N}$ birds. One bird (#5692) showed delayed shedding from day 4 pi
132 to day 6 pi with low viral titres of 250 PFU/ml, 200 PFU/ml, and 50 PFU/ml respectively **(Fig. 2c)**. None
133 of the co-housed ANP32A$^{N129I-D130N}$ sentinel birds were infected **(Fig. 2c)**, suggesting overall that the
134 ANP32A$^{N129I-D130N}$ genotype confers resistance to naturally shed doses of IAV virus.

135 To serologically confirm virus infection, blood samples were collected from all remaining birds on day
136 14 pi and analysed using the haemagglutination-inhibition (HI) assay to detect antibodies to the H9N2
137 IAV. All directly inoculated WT birds (6 of 6 birds) seroconverted with HI titres ranging from 128 to
138 2048 HI units (**Supplementary Fig. 10a**). All WT sentinel birds (6 of 6 birds) seroconverted and had HI
139 titres equal or greater than 128 HI units, confirming extensive virus transmission in the WT isolator. In
140 contrast, the directly inoculated ANP32A$^{N129I-D130N}$ birds (5 of 6 birds) for which shedding was not
141 observed did not seroconvert. Furthermore, the antibody titre in the single positive directly inoculated
142 ANP32A$^{N129I-D130N}$ bird (#5692) was 64 HI units which corroborates the low level of oropharyngeal virus
143 shedding observed in this bird. None of the ANP32A$^{N129I-D130N}$ sentinel birds (6 of 6 birds) seroconverted
144 (**Supplementary Fig. 10b**).

145

146 **ANP32A$^{N129I-D130N}$ chickens display resilience to high-dose IAV infection**

147 We next assessed susceptibility and transmission following challenge with a higher dose of IAV. 10 WT
148 and 10 ANP32A$^{N129I-D130N}$ 2-week-old chickens were intranasally inoculated with 10^6 PFU of H9N2-UDL
149 virus per bird **(Fig. 3a)**, 1000x the dose used in the first experiment. Sentinel chickens of both
150 genotypes were introduced into each isolator 24 hours post-inoculation to assess onward virus
151 transmission **(Fig. 3a)**. 4 ANP32A$^{N129I-D130N}$ and 8 WT sentinel birds were introduced into the WT
152 isolator, while 4 WT and 8 ANP32A$^{N129I-D130N}$ birds were introduced into the ANP32A$^{N129I-D130N}$ isolator.
153 4 directly inoculated birds from each isolator and 4 sentinel birds of the same genotype were sacrificed
154 on day 3 pi or pe respectively for post-mortem examination. Despite the higher dose used for
155 inoculation, no clinical signs or post-mortem pathological lesions were observed in any of the birds.

156 All WT birds were robustly infected and transmitted virus to all WT sentinels (**Fig. 3b**). Infectious virus
157 was detected in swabs from directly inoculated WT chickens from day 1 pi until day 4 pi, peaking on
158 day 2 pi, with a mean titre of 2.9×10^4 PFU/ml (**Fig. 3b**). All WT sentinel birds acquired infection and
159 shed high titres of infectious virus (mean peak titre of 1.3×10^4 PFU/ml on day 2 pe)) from day 1 pe
160 until day 5 or 6 pe (**Fig. 3b)**. In contrast, none of the 4 ANP32A$^{N129I-D130N}$ birds housed in the WT isolator
161 became infected suggesting they were resistant to infection by a naturally transmitted dose (**Fig. 3b**).

162 Low-level and sporadic oropharyngeal shedding of infectious virus was observed in 5 of the 10 directly
163 inoculated ANP32A$^{N129I-D130N}$ birds between day 2 pi and day 7 pi (**Fig. 3c**). 3 of the 10 directly
164 inoculated ANP32A$^{N129I-D130N}$ birds (#941, #946 and #950) shed virus on day 2 and day 3 pi after which
165 4 birds, including bird #941, were randomly culled (**Fig. 3c**). Oropharyngeal shedding was subsequently
166 observed in 2 other GE birds (#947 and #949) between day 4 and day 7 pi (**Fig. 3c**). The daily shed virus
167 titres in infected directly inoculated ANP32A$^{N129I-D130N}$ birds were below 150 PFU/ml and generally
168 more than 2 logs lower than that observed in WT birds except in a single bird (#941) which had a titre
169 of 1.2 x10^3 PFU/ml on day 2 pi. The median duration of infectious virus shedding by the 5 infected
170 directly inoculated ANP32A$^{N129I-D130N}$ birds was 2 days compared to 4 days for the WT birds
171 (**Supplementary Fig. 11**). The area-under-curve (AUC) of shed infectious virus was significantly
172 reduced (p = 0.0018, two tailed T-test) for directly inoculated ANP32A$^{N129I-D130N}$ birds (185.2 PFU/ml ±
173 264.7 PFU/ml SEM) compared to WT birds (5.9 x 10^4 PFU/ml ± 3.7 x 10^4 PFU/ml SEM) (**Supplementary**
174 **Fig. 12**).

175 Sporadic and low-level virus shedding from directly inoculated ANP32A$^{N129I-D130N}$ birds resulted in virus
176 transmission to a single (1 of 4 birds) WT sentinel bird (bird #923), in which oropharyngeal virus
177 shedding was detected on day 5 pe and day 6 pe (80 PFU/ml and 2000 PFU/ml, respectively) (**Fig. 3c**).
178 None of 8 ANP32A$^{N129I-D130N}$ sentinel birds exposed to directly inoculated ANP32A$^{N129I-D130N}$ birds were
179 infected, demonstrating lack of transmission between ANP32A$^{N129I-D130N}$ birds (**Fig. 3c**).

180 Serology confirmed all infections that had been detected virologically. All directly inoculated WT birds
181 and WT sentinel birds housed in the WT isolator seroconverted to the H9N2-UDL virus with HI assay
182 titres ranging from 128 to 4096 HI units (**Supplementary Fig. 13**). In contrast, the HI assay titres for 3
183 of the 4 ANP32A$^{N129I-D130N}$ sentinels in the WT isolator was below the assay detection limit of 5 HI units,
184 while the fourth ANP32A$^{N129I-D130N}$ sentinel had a low antibody titre of 32 HI units, however, infectious
185 virus was not isolated from this bird. HI titres in the directly inoculated ANP32A$^{N129I-D130N}$ birds (ranging
186 from 4 to 256 HI units) were significantly lower than in directly inoculated WT birds (ranging from 256
187 to 4096 HI units), reflecting a lower level of virus replication in these birds (**Supplementary Fig. 13**).
188 Antibodies were not detected in sera from any of the 4 ANP32A$^{N129I-D130N}$ sentinels nor 3 of 4 WT
189 sentinels in the ANP32A$^{N129I-D130N}$ isolator. The single infected WT sentinel bird (bird #923) in the
190 ANP32A$^{N129I-D130N}$ isolator had a HI titre of 256 HI units.

191 Overall, these results demonstrate that following inoculation with high titres of the H9N2-UDL virus,
192 the ANP32A$^{N129I-D130N}$ genotype suppresses viral infection and significantly limits onward viral
193 transmission to naive in-contact birds.

194

Escape viruses contain mutations in their polymerase genes enabling support of virus replication by the edited ANP32A protein

To determine whether any adaptive mutations had occurred during viral infection in ANP32A$^{N129I\text{-}D130N}$ chickens, oropharyngeal swabs were inoculated into WT chicken eggs to amplify sufficient viral material for sequencing. We sequenced viruses isolated from the 6 directly inoculated and infected ANP32A$^{N129I\text{-}D130N}$ chickens (birds #5692, #941, #946, #947, #949 and #950) and the single WT sentinel chicken (bird #923) that acquired infection from the infected GE birds. Comparative sequence analysis showed the presence of different constellations of non-synonymous changes in the polymerase genes and the NS gene that were not present in the isolates from wildtype birds and were not detected in the virus inoculum **(Table 1)**. In all birds with breakthrough infections, mutations PA-E349K, PA-T639I or PB2-M631L were detected, sometimes in combination. In addition, various NS gene mutations and PB1 mutations were detected in isolates from later time points pi from 3 birds, always in combination with PA and/or PB2 mutations.

To assess the functional relevance of the dominant PA and PB2 mutations, we performed minigenome replication assays in ANP32A, B, and E triple-knockout human cells, complementing polymerase function with expression of either wildtype chicken ANP32A (chANP32A^{WT}) or the modified chicken ANP32A (chANP32A$^{N129I\text{-}D130N}$) or the usually non-functional chicken ANP32B or E (chANP32B, chANP32E) proteins. Wildtype chicken ANP32A efficiently supported the activity of all the mutant IAV polymerases **(Fig. 4a)**, indicating that these mutations did not diminish the interaction of wildtype ANP32A protein with the viral polymerase. As expected, chicken ANP32A$^{N129I\text{-}D130N}$ did not support the activity of the wildtype polymerase. However, chicken ANP32A$^{N129I\text{-}D130N}$ supported robust activity of polymerase harbouring the PA-E349K mutation alone, and when this PA mutation was combined with PB2-M631L, the polymerase activity with the edited chicken ANP32A protein was higher than with wildtype chicken ANP32A. The chicken ANP32A$^{N129I\text{-}D130N}$ protein also supported significant activity of polymerases with the combinations of PB2-M631L together with PA-T639I or PA-Q556R. Expression of chicken ANP32B or chicken ANP32E also supported very low levels of activity from the PB2 M631L-PA E349K polymerase **(Fig. 4a)**.

Several of the polymerase mutations, PB2-M631L, PA-E349K, and PA-Q556R, have previously been reported to enhance polymerase activity and replication of mouse-adapted human and avian IAVs[20–24]. PB2-M631L, PA-E349K and PA-Q556R mutations have been detected in IAVs in bird populations and often in avian viruses that infected humans, suggesting that they are human-adaptive mutations[25]. With this in mind, we tested whether they would also enable support of polymerase function by human ANP32A or ANP32B. This was the case as PB2-M631L and PA-E349K substitutions alone or in

228 combination were almost as potent as the common PB2 mutation, E627K, at activating support of the
229 avian influenza polymerase by the shorter mammalian ANP32 proteins in the minigenome replication
230 assay (**Fig. 4b**).

231 To understand the implications of the minigenome replication assay results in the context of whole
232 virus, we generated a recombinant virus that harboured the PA-E349K and PB2-M631L mutations
233 through reverse genetics (RG), and compared its replicative fitness with that of the isogenic RG
234 wildtype H9N2-UDL virus in embryonated chicken eggs and in primary human airway epithelial (HAE)
235 cells grown at air-liquid interface. We infected WT eggs with each virus and observed that viral yields
236 were similar at 12-, 24- and 36-hour timepoints (**Fig. 4c**). In primary cultures of HAE cells, individual
237 growth curves also showed no difference in replication at 24-, 48- and 72-hour timepoints (**Fig. 4d**).

238 Subsequently, we co-infected WT eggs with RG wildtype and double mutant PA-E349K PB2-M631L
239 viruses to assess the competitive fitness of the mutant virus. A minority input of the double mutant
240 virus (<10%) was used to see if it would outcompete the wildtype virus. The double mutant maintained
241 a frequency of 5-10% throughout, demonstrating that the polymerase mutations provided neither a
242 fitness advantage nor a defect (**Fig. 4e**), in line with their equal activity supported by wildtype
243 chANP32A in the polymerase assay (**Fig. 4a**). However, in human airway epithelial cultures, a higher
244 original input of <20% double mutant genomes became enriched to approximately 50% of the virus
245 population by 48 hours post-infection demonstrating a subtle replicative advantage (**Fig. 4f**), also in
246 line with the enhanced support of the mutant polymerase by human ANP32A and B proteins in the
247 polymerase assay (**Fig. 4b**). It is important to note that polymerase gene mutations alone are not
248 sufficient to adapt an avian influenza virus to humans. A major species barrier for avian influenza also
249 exists at the level of cell binding and entry that is determined by the virus haemagglutinin protein, HA.
250 Adaptations in HA are absolutely required for efficient infection and onwards transmission in humans[1].
251 Thus, despite the double mutant H9N2-UDL virus demonstrating a minor fitness advantage over
252 wildtype virus in the HAE competition model, its replication was still 2-3 orders of magnitude lower in
253 human airway cells than that of a human-adapted virus, A/England/195/2009(H1N1), at all timepoints
254 in head-to-head growth kinetics (**Fig. 4d**).

255

256 **Most polymerase mutations that arose in ANP32A$^{N129I\text{-}D130N}$ edited birds are distal to the interface**
257 **with ANP32 amino acids 129 and 130 in the polymerase/ANP32 complex structure**

258 To assess the structural context of the mutations identified in viral isolates from breakthrough
259 infections, we mapped the location of the substituted amino acids in the polymerase subunits to the

published structure of the asymmetric influenza C virus (ICV) polymerase dimer in complex with chicken ANP32A[12] **(Fig. 4g)**. Chicken ANP32A residues N129 and D130 sit on the edge of the fifth leucine-rich repeat domain (LRR5) of ANP32A. One of the polymerase substitutions, PA-T639I, is located in a region of PA in the encapsidating polymerase opposite LRR5 but is not a contact residue with amino acids 129 or 130 in the ICV complex. Another of the PA substitutions, at amino acid 556 is also situated in the encapsidating polymerase in the vicinity of the central region of ANP32A essential for supporting polymerase activity[10,26]. The PB2 residue 631 is located close to the prototypic host-range determining residue 627 and within the PB2-627 domain which is thought to interact with the unstructured LCAR of ANP32A[27]. However, neither of these PA substitutions or PB2 M631L on their own enabled use of the edited ANP32A$^{N129I\text{-}D130N}$ **(Fig. 4a)**.

In contrast, the PA substitution E349K, which had the largest effect on polymerase activity, is not located in any polymerase region interacting with the host ANP32A protein in the solved asymmetric dimer complex (**Fig. 4g**)[12]. However, influenza virus polymerase also forms an alternative symmetric dimer required for the replication of the vRNA genome from a cRNA template, a part of the replication cycle functionally associated with ANP32 proteins, although currently no structure of this dimer in complex with the host protein exists[28,29]. Interestingly, three PA amino acids associated with the breakthrough viruses, 345, 349 and 556, are located on the interface of this symmetric dimer **(Fig. 4h)**[29,30]. How mutations that affect the symmetric dimer might compensate for suboptimal ANP32 proteins to support replication is not currently understood. Since the polymerase in infected cells exists as at least two different conformations, mutations that destabilize one might enable formation of the other even under suboptimal conditions[31].

The PA-349K PB2-M631L double mutant H9N2-UDL escape virus can replicate in chicken embryos lacking ANP32A

Apparently, H9N2-UDL virus adapted *in vivo* to utilize the edited ANP32A$^{N129I\text{-}D130N}$ protein. We asked whether complete removal of ANP32A would eliminate viral escape. We generated male and female surrogate host chickens producing gametes derived from injected PGCs containing a large loss-of-function deletion in ANP32A (AKO) (**Fig. 5a; Supplementary Fig. 14**)[16]. Mating of these surrogate chickens generated homozygous ANP32A-knockout (AKO) eggs and chicks in a single generation**.** We monitored the development of the AKO chick embryos and observed no developmental defects. We hatched a small cohort of sixteen AKO chicks (generation G1 comprising 9 females and 7 males) and did not observe any differences in external appearance, behaviour, internal anatomy, or lay rate (6.22 eggs/week AKO hens; 6.71 eggs/week WT hens) in comparison to wildtype (WT) controls. However,

292 AKO chicks weighed slightly less than wildtype chicks, possibly due to a difference in initial egg size in
293 the WT and AKO eggs (**Supplementary Fig. 15)**.

294 We assessed the robustness of the AKO edit by infecting 11 day-old embryonated chicken eggs which
295 are highly permissive to influenza infection. First, we inoculated ANP32A$^{N129I-D130N}$ GE eggs with 100
296 PFU of wildtype H9N2-UDL (H9N2-UDL^{WT}) virus and observed a low level of viral growth in all GE eggs
297 (**Fig. 5b**). Sequencing of the virus again revealed polymerase mutations (PA-G634E, PA-K635E, PA-
298 K635Q, PB2-G74S, PB1-F185L) in viruses isolated from the ANP32A$^{N129I-D130N}$ GE embryonated eggs.
299 This further confirmed that the N129I-D130N substitution in chicken ANP32A does not completely
300 abrogate virus replication and leads to IAV escape and evolution. In contrast, infection of AKO eggs
301 with 100 PFU of H9N2-UDL^{WT} virus led to no viral replication. Following inoculation with 1000 PFU of
302 H9N2-UDL^{WT}, 2/5 AKO eggs supported a low level of replication (**Fig. 5c**). In contrast, the H9N2-UDL
303 escape double mutant virus replicated in AKO eggs following inoculation at both low (100 PFU) or high
304 (1000 PFU) doses (**Fig. 5c**). This was also the case for the single E349K mutant virus and an independent
305 sample of double mutant virus derived from a plaque pick from the original chicken isolate
306 (**Supplementary Fig. 16**). Together these data indicate that even the entire deletion of chicken
307 ANP32A is not sufficient to abrogate IAV mutant infections of chicken.

308

309 **IAV WT and mutants do not replicate in chicken cells lacking ANP32A, B, and E**

310 We and others previously reported that neither chicken ANP32B nor chicken ANP32E supports IAV
311 polymerase even for viruses with mammalian adapting mutations[10,11,13]. However, we reasoned that
312 the E349K-M631L double mutant virus that replicated in AKO eggs (**Fig. 5c**) may have adapted to use
313 another member of the ANP32 protein family. Indeed, the minireplicon assay had revealed significant
314 but very low activity supported by chANP32B or chANP32E (Fig. 4a). To investigate this possibility,
315 we targeted chicken PGCs to generate cell lines containing concurrent loss-of-expression mutations in
316 ANP32A and ANP32B (AKO/BKO cell line) or in ANP32A and ANP32E (AKO/EKO cell line) or in all three
317 ANP32 proteins (TKO cell line) (**Fig. 5d; Supplementary Fig. 17**). Minireplicon assays indicated that the
318 activity of the H9N2-UDL double mutant polymerase was significantly reduced but detectable in
319 chicken cells lacking only ANP32A (AKO cells), and also in chicken cells expressing only ANP32E
320 (AKO/BKO cells) (**Fig. 5e**). Importantly, polymerase activity was completely absent in TKO cells lacking
321 all three ANP32 proteins.

322

323 Since other viral proteins such as NS1 and NEP are not expressed in minireplicon assays but have
324 effects on polymerase activity and can compensate for defective replication, we tested whether the
325 TKO cells remained resilient to virus replication even when these other viral products were present[32–
326 34]. We first attempted to perform replication assays in TKO cells using the wildtype and double mutant
327 H9N2-UDL virus, however, H9N2 viral replication in *in vitro* cell lines was extremely low. We therefore
328 infected the edited cells with a PR8 recombinant virus harbouring the polymerase and other internal
329 genes from the highly pathogenic H5N1 avian influenza virus A/turkey/Turkey/1/2005 (Tky05). Virus
330 replication was evident by 48 hours post infection in wildtype and all edited cells except the TKO line
331 which yielded no infectious virus even after 120 hours incubation (**Fig. 5f**). Finally, we infected the set
332 of edited cell lines with a representative of the contemporary highly pathogenic H5N1 clade 2.3.4.4b
333 viruses, and confirmed complete absence of replication in the TKO cells (**Fig. 5g**). Taken together, our
334 result implies that single edits or deletions of single ANP32 proteins is not sufficient to generate
335 influenza resistant birds but that edits of all three members of the ANP32 family will be needed.

336

337 **Discussion**

338 Breeding for resistance and resilience to disease has significant potential in farmed poultry[35,36]. The
339 production of transgenic chickens that expressed an RNA decoy that inhibits the IAV polymerase and
340 prevented onward viral transmission to neighbouring birds was the first demonstration that genetic
341 engineering could be used to introduce resistance to infectious diseases in chicken[37]. With recent
342 advances in the development of genome editing technology, novel resistance/resilience alleles can
343 now be introduced into chicken populations by editing host genes essential for pathogenic infections
344 to specifically abrogate their pro-viral functions[38].

345 Here we introduced a specific gene edit to the host protein ANP32A that had been shown to abrogate
346 its support for the influenza polymerase in cell culture[10,13]. GE birds carrying this edit showed no
347 adverse health or productivity effects and were resistant to IAV infection by a natural transmission
348 route following exposure to other infected birds. Thus, our data show the promise of this strategy for
349 mitigating the incursion of avian influenza into farmed poultry from wild bird sources. Even following
350 a direct inoculation with 10^3 infectious virus particles, only a single bird was infected and the viral
351 titres shed were low and transient, and no onwards transmission occurred. However, following direct
352 inoculation with a higher dose, breakthrough infection occurred in the GE birds. Influenza virus is
353 notorious for its ability to evolve, and we detected a series of different amino acid substitutions in the
354 viral polymerase genes of viruses isolated from the GE chickens that had enabled adaptation of the
355 enzyme to co-opt support from the edited ANP32A protein, and also to utilize otherwise suboptimal

356 ANP32 family members. These mutations unexpectedly allowed the usually host-restricted avian
357 influenza polymerase to use the shorter human ANP32A and B and thus partially adapted the viral
358 polymerase for replication in mammals. Although unintended, this consequence clearly indicates the
359 importance of a robust genome editing strategy and subsequent appraisal that includes challenge with
360 multiple avian influenza genotypes at non-physiological exposure levels to rule out the opportunity
361 for adaptive viral evolution.

362 We further generated chickens that entirely lacked expression of ANP32A, but the wildtype virus still
363 replicated at low levels in some of the eggs, and the mutant virus was only marginally restricted.
364 Finally, we edited all three members of the ANP32 family to generate chicken cells lacking their
365 expression, and found no virus polymerase activity, even of the mutant polymerase, and no
366 breakthrough infection in these cells. This combination of knockouts is expected to be deleterious to
367 the animals' health, but illustrates a proof of principle that multiple edits in host genes could be
368 combined to confer sterile resistance. Indeed, editing of the three ANP32 genes will be futile if
369 increased resistance to Avian Influenza is accompanied by any loss in fitness of the birds; for example
370 effects on development, weight gain or fecunidity, and/or increased susceptibility to other avian
371 pathogens.

372 Future assessment of GE animals, after the research phase of their development and prior to their
373 distribution, should take into account whether appropriate investigatory steps have been carried out
374 to evaluate if genome edited livestock might drive pathogen evolution. This is especially relevant for
375 pathogens with zoonotic potential as was shown here. We suggest that a suitable strategy for
376 generating avian influenza resistant chickens will require multiple edits that destroy the pro-viral
377 potential of ANP32A, B and E to eliminate the likelihood that escape mutants can arise.

378

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Authors' contribution

AIA, DHG, CMS, DB, JQ, KS, CC, SR LT, JB, AS, SN, MK, HS, MJM, WSB contributed to production of experimental data. AIA, DHG, CMS, DB, JQ, KS, CC LT, JB, AS, SN, MK, HS, MJM, JL, HMS WSB contributed to experimental design and interpretation of the data. All authors contributed to writing of the manuscript.

Ethics declaration

Competing interests

The author MJM is inventor on patent application WO 2020074915 for the iCaspase9 surrogate host chicken. The University of Edinburgh is the applicant. The authors WSB, MJM, HMS, DHG, CMS, AIA are inventors on a patent application for the work presented in this manuscript. The University of Edinburgh is the applicant. The remaining authors declare no competing interests.

The data supporting the findings of this study are available within the article and its Supplementary Information. The source data for the main figures and extended data figures are provided as Source Data files. Illumina RNA sequencing data for PGC transcriptome analysis are deposited in the GEO and SRA archives at NCBI (Accession number GSE182397). The authors declare that all unique materials used are readily available from the authors upon MTA agreement.

413 **Methods**

414 **Animals**

415 Fertile eggs were obtained from commercial Hy-line layer flocks bred at the National Avian Research
416 Facility, Midlothian, United Kingdom. All chicken lines were bred and maintained under UK Home
417 Office License. All experiments and procedures were performed in accordance with relevant UK Home
418 Office regulations. Experimental protocols and studies were reviewed by the Roslin Institute Animal
419 Welfare and Ethical Review Board (AWERB) Committee, GM and Biological Safety Committee and
420 performed under Home Office Licence (PP9565661). All animal challenge work was approved and
421 regulated by the UK government Home Office under the project license (P68D44CF4) and reviewed by
422 the Pirbright Animal Welfare and Ethics Review Board (AWERB). All personnel involved in the
423 procedures were licensed by the UK Home Office. All procedures were performed in accordance with
424 these guidelines and the study is reported in line with the ARRIVE guidelines.

425

426 **PGC derivation and culture**

427 PGC lines were derived from individual fertile eggs, cultured in FAOT medium and expanded to
428 400,000 cells in 5 weeks before performing gene editing experiments[39]. Fertile eggs bred from Hy-line
429 layer lines were incubated for 2.5 days and then 1µl of embryonic blood was taken from the dorsal
430 aorta of HH stage 16 HH embryos and placed into FAOT medium. FAOT medium contains custom-
431 made Avian Knockout DMEM (Life Technologies #041-96570M) 1× B-27 supplement (Life Technologies
432 #17504044), 2.0 mM GlutaMax (Life Technologies #35050-038), 1× non-essential amino acids (Life
433 Technologies #11140050), 1× EmbryoMax nucleosides (Merck Millipore #ES-008-D), 0.1 mM β-
434 mercaptoethanol (Life Technologies #31350010), 0.2% ovalbumin (Sigma-Aldrich A5503), 1.2 mM
435 sodium pyruvate (Life Technologies #11360070), 0.15 mM CaCl2, 0.01% sodium heparin, 4 ng/ml h-
436 FGF2 (R&D Systems), 50 ng/ml ovotransferrin (Sigma-Aldrich C7786) and 25ng/ml activin A
437 (Peprotech). PGCs were grown at 37 °C in a 5% CO_2 atmosphere and fed every 48 hours.

438

439 **CRISPR Plasmids and ssODN donor**

440 PX458-mcherry, PX458-GFP**,** PX459 V2.0 and HF-PX459 V 2.0 vectors were used for expression of
441 CRISPR/Cas9[14,15,40]. PX458-mcherry was a gift from Joanna Wysocka (Addgene plasmid # 161974).
442 PX458 was a gift from Feng Zhang (Addgene plasmid # 48138). PX459 V2.0 vector was a gift from Feng
443 Zhang (Addgene plasmid # 62988). gRNA sequences were selected using CHOPCHOP gRNA design web
444 tool (http://chopchop.cbu.uib.no/) which also generated potential off-target sites for the selected
445 gRNA[41]. gRNA oligonucleotides were synthesized by Invitrogen and inserted into PX459 V2.0 and HF-
446 PX459 V2.0 vectors using methods previously described[14]. Short single-stranded oligonucleotide DNA
447 (ssODN) donor was an Ultramer® DNA Oligonucleotide synthesized by Integrated DNA Technologies
448 (IDT). gRNA and ssODN sequences are listed in **Supplementary Table 3**.

449

450 **PGC gene editing and DNA sequencing**

451 To generate $ANP32A^{N129I-D130N}$ cells, PGCs were transiently transfected with 1.5 µg of PX459 V2.0
452 CRISPR/Cas9 vector and 10 µM of ssODN donor using Lipofectamine 2000 (Life Technologies) to target
453 exon 4 of ANP32A. This was followed by treatment with 0.1 mg/ml puromycin (Sigma-Aldrich #P7255)
454 to enrich for transfected cells[15]. To generate $ANP32A^{knockout}$ cells, chicken PGCs were transiently

455 transfected with 2.0 µg of PX459 V2.0 CRISPR/Cas9 vector using Lipofectamine 2000 transfection
456 reagent to target exon 1 of ANP32A[10]. To generate the AKO genotype containing a 15-kb loss-of-
457 function deletion in ANP32A, chicken PGCs were transiently transfected with 1.5 µg of PX459 V2.0
458 CRISPR/Cas9 vector to target exon 1 and intron 5 of ANP32A and followed by treatment with 0.1
459 mg/ml puromycin (Sigma-Aldrich #P7255) to enrich for transfected cells[15]. Single cell cultures of
460 puromycin-selected cells were subsequently established to isolate clonal populations of homozygous
461 gene-edited PGCs for downstream experiments as described previously[15]. Briefly, PGCs were seeded
462 at 1 cell per well in 110 µl FAOT medium in 96-well plates using a FACS Aria III machine (BD Biosciences)
463 and subsequently cultured for 2 to 3 weeks when cell density will reach 30 to 50%. The PGC cultures
464 were then transferred to 48-well plates and subsequently into 24-well plates for further expansion for
465 downstream experiments. Alternatively to generate the AKO genotype, PGCs were transiently
466 transfected with 1.5 µg each of PX458-GFP and PX458-mcherry CRISPR/Cas9 vectors to target exon 1
467 and intron 5 of ANP32A and followed by fluorescence-activated cell sorting (FACS) 48 hours later to
468 establish single cell cultures to isolate clonal populations of homozygous gene-edited PGCs. To
469 generate the BKO genotype containing a 134-bp loss-of-function deletion in ANP32B, PGCs were
470 transiently transfected with 1.5 µg each of PX458-GFP and PX458-mcherry CRISPR/Cas9 vectors to
471 target the promoter region and exon 1 of ANP32B, followed by fluorescence-activated cell sorting
472 (FACS) 48 hours later to establish single cell cultures to isolate clonal populations of homozygous gene-
473 edited PGCs. To generate the EKO genotype containing a 160-bp loss-of-function deletion in ANP32E,
474 PGCs were transiently transfected with 1.5 µg each of PX458-GFP and PX458-mcherry CRISPR/Cas9
475 vectors to target exon 2 and intron 2 of ANP32E, followed by fluorescence-activated cell sorting (FACS)
476 48 hours later to establish single cell cultures to isolate clonal populations of homozygous gene-edited
477 PGCs. To generate the AKO/BKO (containing concurrent loss-of-function deletions in ANP32A and
478 ANP32B) and AKO/EKO genotypes (containing concurrent loss-of-function deletions in ANP32A and
479 ANP32E), BKO PGCs and EKO PGCs were respectively transiently transfected with 1.5 µg each of
480 PX458-GFP and PX458-mcherry CRISPR/Cas9 vectors to target exon 1 and intron 6 of ANP32A and
481 followed by fluorescence-activated cell sorting (FACS) 48 hours later to establish single cell cultures to
482 isolate clonal populations of homozygous gene-edited PGCs. To generate the TKO genotype
483 (containing concurrent loss-of-function deletions in ANP32A, ANP32B and ANP32E), AKO/BKO PGCs
484 were transiently transfected with 1.5 µg each of PX458-GFP and PX458-mcherry CRISPR/Cas9 vectors
485 to target exon 2 and intron 2 of ANP32E, followed by fluorescence-activated cell sorting (FACS) 48
486 hours later to establish single cell cultures to isolate clonal populations of homozygous gene-edited
487 PGCs.

488

489 To screen for $ANP32A^{N129I-D130N}$ cells, single cell clones were analysed by PCR amplification of genomic
490 DNA and Sanger sequencing using primers (5' – AGAGGAAGGGAGCAAAAGTCA – 3', 5' –
491 ATGCTTGTCTTCCTCCTTCCA – 3'). To screen for $ANP32A^{knockout}$ cells containing targeting of exon 1 only,
492 single cell clones were analysed by PCR amplification of genomic DNA and then cloning of the PCR
493 products into pGEM-T Easy vector (Promega), followed by Sanger sequencing using T7 promoter
494 forward primer. To screen for the AKO genotype, single cell clones were analysed by PCR amplification
495 of genomic DNA and Sanger sequencing using primers (5' – TCAAAGTCCCTTATTACCGCG – 3', 5' –
496 CCTTTCACTCCCCATCTTTCA – 3') that bind to areas outside the deleted 15-kb region and amplify a PCR
497 product of approximately 220 bp only if the deletion is successful but will fail to yield a product if there
498 is no deletion. To screen for the BKO genotype, single cell clones were analysed by PCR amplification
499 of genomic DNA and Sanger sequencing using primers (5' – GGTGCCATTTTGTCGAGGG – 3', 5' –
500 CTCTCCAGGCTTCTTGTTGC – 3') overlapping the deleted region. To screen for the EKO genotype, single
501 cell clones were analysed by PCR amplification of genomic DNA and Sanger sequencing using primers

(5' – ATGTCATGGAGGCGCAGT – 3', 5' – CCCCAAATCAGTAAAAGCCCC – 3') overlapping the deleted region. PCR primers used for amplification of selected off-target sites are detailed in **Supplementary Table 4.** PCR gel electrophoresis data was collected using the NuGenius Gel documentation system (Syngene). Sequencing data was viewed and analysed using SeqMan Pro 17 and MegAlign Pro 17 (Lasergene 17, DNASTAR) or SnapGene Version 6.2.1(Dotmatics).

PGC transcriptome analysis

200,000 PGCs were expanded in FAOT culture medium to 2 x 10^6 cells. PGCs were subsequently pelletised by centrifugation, resuspended in phosphate buffered saline (PBS) and then pelletised again. RNA was purified from the washed cell pellet using the Qiagen RNeasy Mini kit (Qiagen 74104) according to the manufacturer's instructions. RNA concentration was measured using the NanoDrop® Spectrophotometer (Thermo Scientific ND-1000). RNA quality was assessed using the Agilent RNA 6000 Nano Kit (Agilent Technologies 5067-1511) and the 2100 Bioanalyzer (Agilent Technologies G2939BA). All the RNA samples used in RNA sequencing (RNA-seq) had an RNA integrity number (RIN) ranging from 8.0 to 10.0

cDNA libraries were constructed by the Beijing Genomics Institute (BGI, Hong Kong, China). Briefly, poly-A containing mRNA molecules were purified using poly-T oligo-attached magnetic beads (New England Biolabs) that were subsequently fragmented into shorter mRNA fragments using divalent cations under an elevated temperature (Ambion® RNA fragmentation reagents kit, Thermo Fisher Scientific). Reverse transcriptase and a random primer (Invitrogen) were then used to synthesize First strand cDNA from the cleaved RNA fragments. Second strand cDNA synthesis proceeded with a DNA Polymerase I (New England Biolabs) and RNase H (Invitrogen), and the RNA template was removed. An 'A' base was added to the resulting cDNA fragments, that was subsequently ligated to an adapter. Finally, the products were enriched by PCR before purification with the MinElute PCR Purification Kit (Qiagen). This purified cDNA library was used in RNA-Seq analysis.

RNA-seq reads were generated by BGI using the Illumina Novaseq 6000 system. Following sequencing, initial analysis was conducted by BGI using SOAPnuke software (developed by BGI) to filter the raw sequence reads using the following parameters: - n 0.01 -l 20 -q 0.4 -A 0.25 --cutAdaptor -Q 2 -G --polyX 50 --minLen 150 -i. An entire read was removed if 25% of its sequence matched the adaptor sequence. To filter low-quality data, an entire read was removed if more than 50% of its bases had a quality value lower than 20. Also, an entire read was deleted if the frequency of unknown bases was greater than 5%. The remaining filtered reads (over 78 million per sample), defined as 'clean reads' were stored in FASTQ format. FASTQ files were imported into the CLC Genomics Workbench v20.0.4 (Qiagen) for quality-control processing and analysis. RNA-seq data are deposited in the GEO and SRA archives at NCBI (Accession number GSE182397).

RNA-seq read mapping and DEG analysis was performed as previously described[42,43]. Briefly, RNA-seq reads were subjected to quality trimming before mapping to the ENSEMBL galGal5-annotated assembly (GRCg6a; 15-12-2020) for alignment and quantitative analysis of expression using the 'RNA-Seq Analysis' tool of the CLC Genomics Workbench (CLC Bio v2.18). For quantitative analysis, trimmed mean of M-values normalization (TMM) of the trimmed data set was performed by filtering out genes showing zero or NaN expression values. The fold change and False Discovery Rates (Bonferroni) were calculated using the RNA-Seq Analysis tool while differential expression within the RNA-Seq data was analysed using the Differential Expression for RNA-Seq tool of the CLC Genomic Workbench (CLC Bio v2.2). HEAT Maps were generated from TMM-normalised samples using the 'Create HEAT Map for

546 RNA-Seq' tool in the same suite, using Euclidean Distance and Complete Cluster Linkage. Principal
547 component analysis (PCA) plots were generated from the TMM-normalised samples using the 'PCA
548 for RNA-Seq' tool in the same suite.

549

550 **Generation of GE chickens**

551 Male and female GE PGCs were micro-injected separately into stage 15-16^{+} HH (ED 2.5) surrogate
552 iCaspase9 host embryos as described previously[16]. Briefly, 1.0 µl of 25 mM B/B compound (in DMSO)
553 (Takara Bio) was added to a 50 µl cell suspension (5000 PGCs/µl) and maintained at room temperature.
554 ANP32A$^{N129I-D130N}$: one male (FR5M#7) or three female (FR3F#3, FR3F#40 and FR6F#10) clonal GE PGC
555 lines. 1.0 µl of the B/B compound-PGC mixture was injected into the dorsal aorta of embryos in
556 windowed eggs. Egg shells were sealed with medical Leukosilk tape (BSN Medical) and then incubated
557 until hatch. Surrogate males and female chickens (G0) were mated in pens to produce homozygous
558 GE offspring. To screen for homozygous ANP32A$^{N129I-D130N}$ embryos or chicks, chorioallantoic
559 membrane (CAM) (for embryos) or CAM and blood (from hatchlings) were analysed by PCR
560 amplification of genomic DNA and Sanger sequencing using primers (5' – ACTCCTTTTGTCACGAGAAGC
561 – 3', 5' – TTCCTCCTCATCGTCTAAGCC – 3'). To screen for homozygous AKO embryos, CAM were
562 analysed by PCR amplification of genomic DNA and Sanger sequencing using primers (5' –
563 TCAAAGTCCCTTATTACCGCG – 3', 5' – CCTTTCACTCCCCATCTTTCA – 3') that bind to areas outside the
564 deleted 15-kb region and amplify a PCR product of approximately 220 bp only if the deletion is
565 successful but fail to yield a product if there is no deletion. GE chickens received routine vaccinations
566 and blood samples were sent to Sci-Tech Labs (Cawood Scientific), Dublin, Ireland to perform ELISA
567 tests to assess response to vaccines.

568

569 **Western blot analysis**

570 To analyse ANP32A expression in gene-edited clonal lines (**Supplementary Fig. 3 & Supplementary**
571 **Fig. 17k**), at least 150,000 cells were lysed in 50 µl of 1X RIPA lysis buffer (sc-24948, Santa Cruz
572 Biotechnology) containing protease and phosphatase inhibitor (Halt, Thermo Scientific 78440)
573 according to the manufacturer's instruction. To analyse ANP32A expression in AKO embryos
574 (**Supplementary Fig. 14g**), approximately 2 mg of embryonic tissue was lysed in 100 µl of 1X RIPA lysis
575 buffer (sc-24948, Santa Cruz Biotechnology) containing protease and phosphatase inhibitor (Halt,
576 Thermo Scientific 78440) according to the manufacturers' instruction. Denaturing electrophoresis and
577 western blotting were performed using the NuPAGE electrophoresis system (Invitrogen) following the
578 manufacturer's protocol. Immunoblotting was performed using the following primary antibodies;
579 rabbit anti-ANP32A (Sigma-Aldrich AV40203; 1/1000 dilution) and mouse anti-ɣ-tubulin (Sigma-
580 Aldrich TS6557; 1/1000 dilution). The following secondary antibodies were used: goat anti-mouse
581 IRDye 800CW (LI-COR 925-32211; 1/10,000 dilution) and goat anti-rabbit IRDye 680RD (LI-COR 925-
582 68070; 1/10,000 dilution). Protein bands were visualised through fluorescence using the Odyssey
583 Imaging System (LI-COR) according to the manufacturer's instruction.

584

585 **PGC differentiation into adherent fibroblast-like cells**

586 150,000 PGCs were incubated in 500 µl of high calcium FAOT medium containing 1.8mM $CaCl_2$ in
587 fibronectin-coated or gelatin-coated wells in 24-well plates for 48 hours. Subsequently, the FAOT
588 medium was replaced with PGC fibroblast cell culture medium and then refreshed every 48 hours by

taking out 300 µl and adding back 300 µl of PGC fibroblast medium. Adherent fibroblast-like cells were visible with 48 hours of culture in PGC fibroblast medium. Cell culture medium was refreshed every 48 hours and cells were split 1:3 once they are reached 85-90% confluency in 24-well plates. PGC fibroblast-like cell culture medium contains 10% ES-grade foetal bovine serum (Life Technologies #16141061), 1% chicken serum (Biosera #CH-515), 0.1% 100x NEAA (Life Technologies #11140050), 0.1% sodium pyruvate (Life Technologies #11360070), 0.1% 100x GlutaMax (Life Technologies #35050-038), 50ng/ml ovotransferrin (Sigma-Aldrich C7786) in Knockout DMEM (Life Technologies #10829018). PGC fibroblast cultures were maintained at 37 °C in a 5% CO_2 atmosphere.

Cells and cell culture

Madin-Darby canine kidney (MDCK) cells (ATCC) were maintained in cell culture medium containing Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% foetal bovine serum (FBS) (Life Technologies) and 1% penicillin-streptomycin (Life Technologies) at 37 °C in a 5% CO2 atmosphere. ANP32A-ANP32B-ANP32E-triple-knockout human eHAP1 cells (Horizon Discovery) lacking ANP32 expression were generated by Dr Ecco Staller at the University of Oxford [9]and maintained in Iscove's modified Dulbecco's medium (Thermo Fisher) supplemented with 10% FBS, 1% nonessential amino acids (Life Technologies), and 1% penicillin/streptomycin.

Minigenome replication assay

Influenza polymerase activity was assessed by using the chicken polI minigenome system as described previously.[8,10,44] pCAGGS expression plasmids encoding each polymerase component and NP for H5N1 50–92, H5N1 ty/05 and H9N2-UDL are described previously.[8,44] To measure influenza polymerase activity, PGC fibroblasts were transfected using Lipofectamine LTX (Invitrogen) according to manufacturers' instructions in 24-well plates with pCAGGS plasmids encoding the PB1 (100 ng), PB2 (100 ng), PA (100 ng) and NP (100 ng) proteins, together with 100 ng chicken-specific minigenome reporter, either Empty pCAGGS or pCAGGS expressing chicken ANP32A (100 ng) and, as an internal control, 100 ng Renilla luciferase expression plasmid (pCAGGS-Renilla). Cells were incubated at 37°C, and 48 hours after transfection, cells were lysed with 120 ml of passive lysis buffer (Promega). Firefly and Renilla luciferase bioluminescence were measured using a Dual-luciferase system (Promega) with an EG&G Berthold LB 96 Microplate Luminometer or a Cytation 3 plate reader (Agilent-BioTek).

To assess polymerase activity in ANP32A-ANP32B-ANP32E-triple-knockout eHAP1 cells, cells were transfected using Lipofectamine 3000 (Invitrogen) in 24-wells with pCAGGS expression plasmids encoding H9N2-UDL PB1 (0.04 µg), PB2 (0.04 µg), PA (0.02 µg), NP (0.08 µg), chicken or human-specific Firefly minigenome reporter (0.08 µg) and *Renilla* luciferase expression plasmid (0.04 µg) together with chicken ANP32-FLAG or human ANP32-FLAG or empty expression plasmid (0.04 µg) and incubated at 37˚C for 24 hours. Cells were subsequently lysed in 50 µl passive lysis buffer (Promega) for 30 minutes with gentle shaking at room temperature. Firefly and *Renilla* bioluminescence were measured using the dual-luciferase system (Promega) with a FLUOstar Omega plate reader (BMG Labtech).

***In vivo* challenge of chickens with influenza virus and transmission to naïve sentinels**

Mixed sex commercial Hy-line layer chickens (WT and ANP32A$^{N129I\text{-}D130N}$) were hatched at the National Avian Research Facility, Midlothian, United Kingdom and transported at 1 day of age to The Pirbright Institute, Surrey, UK. Pre-infection sera were obtained from all chickens, and all were negative for reactive influenza antibodies against A/chicken/Pakistan/UDL01/08 (H9N2-UDL) by haemagglutinin inhibition assay (HI). Chickens were housed in two groups according to genotype, WT or ANP32A$^{N129I\text{-}D130N}$. At 7 days of age, chickens to be directly inoculated were housed in negative pressured BioFlex® B50 Rigid Body Poultry isolators (Bell Isolation Systems). At 2-weeks of age, chickens in isolators were inoculated intranasally with 100 µl (50 µl per nare) of H9N2-UDL virus, either at a low dose of virus (1 x 10^3 PFU) or high dose (1 x 10^6 PFU). 24 hours after direct inoculation, naïve sentinel birds were introduced into the isolators to assess transmission. Numbers of birds involved in each experiment are detailed in **Fig. 2** and **Fig. 3**. 14 days post-inoculation, birds were humanely euthanised by intravenous administration of sodium pentobarbital, blood was collected by cardiac puncture and sera collected for analysis of virus specific antibodies by HI. Infection of birds was assessed by swabbing both the oropharyngeal and cloacal cavities daily with sterile polyester tipped swabs (Fisher Scientific, UK) which were transferred into viral transport media, vortexed briefly, clarified and stored at -80 °C prior to virus detection[45].

Plaque assay

Infectious virus titration was by plaque assay on MDCK cells. MDCKs were inoculated with 10-fold serially diluted samples and overlaid with 2% agarose (Oxoid) in supplemented DMEM (1× MEM, 0.21% BSA V, 1 mM L-Glutamate, 0.15% Sodium Bicarbonate, 10 mM Hepes, 1× Penicillin/Streptomycin (all Gibco) and 0.01% Dextran DEAE (Sigma-Aldrich, Inc.), with 2 µg/ml TPCK trypsin (SIGMA). MDCKs were then incubated at 37 °C for 72 hours. Plaques were developed using crystal violet stain containing methanol. The limit of virus detection in the plaque assays was 5 pfu/ml.

Haemagglutination inhibition assay (HI)

Haemagglutinin inhibition (HI) assays were carried out using the challenge virus H9N2-UDL. HI assays were performed according to standard procedures[46]. Samples with titres below 2 HI units were considered negative.

Sequencing of viruses isolated from chicken oropharyngeal swabs

To sequence viruses recovered from directly inoculated and sentinel chickens, 100 µl of clarified oropharyngeal swab sample was inoculated into embryonic day (ED) 10 chicken eggs (Valo BioMedia GmbH) and incubated at 37°C with turning for 72 hours before allantoic fluid was harvested. Viral RNA was extracted from allantoic fluid using RNA extracted from each concentration, using the QIAmp viral RNA minikit (Qiagen) according to manufacturer instructions and virus cDNA produced using SuperScript IV (Invitogen) RT-PCR using the universal primer Optil-R1: GTT ACG CGC CAG TAG AAA CAA GG according to the manufacturer's instructions. PCR amplification of all eight influenza A virus segments was achieved by PCR with a mixture of three universal primers (Optil-F1: GTT ACG CGC CAG CAA AAG CAG G, Optil-F2: GTT ACG CGC CAG CGA AAG CAG G and Optil-R1: GTT ACG CGC CAG TAG AAA CAA GG) and Q5® High-fidelity DNA polymerase (NEB). 1 ng of dsDNA was used to prepare sequencing libraries using the Nextera XT DNA kit (Illumina). Pooled libraries were sequenced on a

672 2x300cycle MiSeq Reagent Kit v2 (Illumina, USA) and analysed using Geneious Prime 2019 software
673 (Biomatters Inc.).

674 **Human airway epithelial cell infection**

675 Triplicate wells of primary human airway epithelial (HAE) cells (purchased from Epithelix Sarl, Inc) at
676 air-liquid interface were washed apically with 200µl serum-free DMEM (Gibco) to remove mucus
677 before being infected with 0.01 PFU/cell of virus for 1 hr. Inoculum was removed and the apical surface
678 washed twice. At timepoints post-infection 200µl serum-free DMEM was added to the apical surface
679 and removed after 10 mins at 37°C to take harvests for plaque assay or variant quantification for
680 competition assay.

681 **Competition assay between UDL WT and mutant viruses**

682 Chicken eggs (n=4) or human airway epithelial cell inserts (n=3) were co-infected with a mixture of
683 viruses and harvests taken at timepoints post-infection. Viral RNA was extracted using MagMAX™
684 Viral/Pathogen kit on KingFisher™ Flex Purification System (Thermo), cDNA generated using
685 Superscript III (Thermo) and Uni-12 primer. A pair of ~200bp amplicons across position 349 of the PA
686 gene and position 631 of the PB2 gene were generated by PCR for each sample and were designed to
687 include one of four 4bp terminal barcodes (CACA, GTTG, AGGA or TCTC) using the primers in
688 **Supplementary Table 5**. The pair of PA and PB2 amplicons for each sample were combined. Pools of
689 samples were made using samples representing each of the four unique barcodes and a second
690 barcode was added to the pooled amplicons using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina
691 (NEB). Pooled samples were pooled together and sequenced with Illumina MiSeq v2 150 PE micro kit
692 (Illumina). Sequencing reads were demultiplexed and variant proportions at each locus quantified.

693

694 **Statistical analysis and graphical illustration**

695 Statistical analysis of biological replicates was performed by unpaired two-tailed T-test, One-way
696 ANOVA with Dunnett's multiple comparison test, or Mann-Whitney test using GraphPad Prism 9.
697 Sample sizes were not predetermined using any statistical methods. Some illustrations in **Fig. 1 and**
698 **Fig.5** were generated using BioRender.com.

699

Figure legends

Fig. 1. Breeding strategy for homozygous ANP32A$^{N129I\text{-}D130N}$ chicken.

a, ANP32A editing strategy: two nucleotide changes (red letters) introduce asparagine (N) position 129 (N129I) and aspartic acid (D) position 130 (D130N) missense mutations. The third nucleotide change (green letters) is a synonymous mutation in the gRNA PAM and serves as a marker control for allelic contribution from the male and female surrogate hosts. **b**, Male and female PGC cultures were derived from the blood of individual chick embryos. The PGCs were edited, and clonal lines of GE PGCs were propagated and analysed. GE PGCs were differentiated into fibroblast-like cells for IAV polymerase assays. To generate GE chicks, GE PGCs were mixed with B/B dimerization compound (to induce cell death of host embryo germ cells) and injected into iCaspase9 host embryos, which were incubated to hatch. After hatching, the surrogate hosts were raised to sexual maturity and directly mated. All offspring from eggs laid by the surrogate hosts were biallelic for the edit and contained the parent-specific PAM nucleotide change. **c**, the activity of reconstituted IAV polymerase was assessed in fibroblast-like cells derived from ANP32A^{knockout} (Knockout), ANP32A$^{N129I\text{-}D130N}$ (N129I-D130N) or wildtype (WT) PGCs. Cells were transfected with avian IAV polymerase (PB2/627E - black bars) or human-adapted isoforms (PB2/627K - grey bars), Firefly minigenome reporter and Renilla reporter control plasmids and then incubated at 37°C for 48 hours. Wild-type chicken ANP32A (chA) cDNA was co-expressed with minigenome plasmids to rescue polymerase activity in ANP32A$^{N129I\text{-}D130N}$ cells. Data shown are Firefly activity normalised to Renilla plotted as mean ± SEM derived from (n=3) three independent experiments each consisting of three technical replicates. Error bars represent standard error of mean (SEM). One-way ANOVA and Dunnett's multiple comparison test were used to compare polymerase activity in the GE cells with polymerase activity in WT cells. Unpaired two-tailed t-test was used to compare ANP32A$^{N129I\text{-}D130N}$ and ANP32A$^{N129I\text{-}D130N}$ +chA data. Statistical annotations are defined as **=P≤0.05, ****=P≤0.0001*. **d**, Image*:* wild-type (WT) hen (*left*) and homozygous ANP32A$^{N129I\text{-}D130N}$GE hen (*right, blue ring on right shank*).

Fig 2. Assessment of low-dose IAV infection in ANP32A$^{N129I-D130N}$ chickens.

a, Schematic of low-dose *in vivo* challenge of 2-week-old chickens with H9N2-UDL influenza A virus (A/chicken/Pakistan/UDL01/08). Chickens were housed in negative pressure poultry isolators. Prior to challenge all birds were bled from the wing vein to obtain pre-infection sera. Groups of ten WT (black) chickens or ten ANP32A$^{N129I-D130N}$ (white) chickens were intranasally inoculated with 1 x 10^3 PFU of H9N2-UDL virus per bird. Uninoculated sentinel chickens were introduced into the isolators 24 hours post infection to assess for transmission from the directly inoculated birds. Oropharyngeal cavities of each bird were swabbed daily from the day of inoculation (D0) until day 7 (D7) post-inoculation. Infectious virus titre in swabs was measured by plaque assay on MDCK cells **(b,c)**. **c,** Bird ID number for directly inoculated ANP32A$^{N129I-D130N}$ birds above the detection limit is indicated. *DL – detection limit of 10 PFU/ml for plaque assay.*

751 **Fig. 3: Assessment of high-dose IAV infection in ANP32A$^{N129I-D130N}$ chickens**.

752 **a**, Schematic of high-dose *in vivo* challenge of 2-week-old chickens with H9N2-UDL influenza A virus
753 (A/chicken/Pakistan/UDL01/08). Chickens were housed in negative pressure poultry isolators. Prior to
754 challenge all birds were bled from the wing vein to obtain pre-infection sera. Groups of ten WT (black)
755 chickens or ten ANP32A$^{N129I-D130N}$ (white) chickens were intranasally inoculated with 1 x 10^6 PFU of
756 H9N2-UDL virus per bird. Uninoculated naive sentinel chickens were introduced into the isolators 24
757 hours post challenge (day 1 pi) to assess for transmission from the directly inoculated birds.
758 Oropharyngeal cavities of each bird were swabbed daily from the day of inoculation (day 0) until day
759 7 post inoculation. Infectious virus titre in swabs was measured by plaque assay **(b,c)**. **c,** Bird ID
760 number for plaque-positive directly inoculated ANP32A$^{N129I-D130N}$ birds are indicated**.** Bird #941
761 was one of four directly inoculated ANP32A$^{N129I-D130N}$ birds culled on day 3 pi for post-mortem
762 examination. *DL – detection limit of 10 PFU/ml for plaque assay.*

Fig. 4. Assessment of mutations identified in polymerase genes of viruses isolated from infected ANP32A$^{N129I\text{-}D130N}$ chickens.

a-b, Influenza A virus (H9N2-UDL) polymerase harbouring single or combinations of PA and PB2 mutations detected in virus isolated from ANP32A$^{N129I\text{-}D130N}$ chickens was reconstituted together with NP by plasmid transfection in eHAP1 human cells that lack ANP32 expression and complemented with chicken (**a**) or human (**b**) ANP32-FLAG proteins. Polymerase activity was measured at 24 hours post-transfection by Firefly luciferase signal generated from a minireplicon and a Renilla luciferase transfection control. Data shown are Firefly activity normalised to Renilla plotted as mean ± SEM derived from three (n=3) independent experiments, each consisting of three technical replicates. . Error bars represent SEM. Data statistically analysed by one-way ANOVA and Dunnett's multiple comparison test to determine polymerase constellations whose activity varied from wildtype H9N2-UDL polymerase. Statistical annotations are defined as **=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001*.

c, WT embryonated eggs were inoculated with 100 PFU of wildtype H9N2-UDL (H9N2-UDL^{WT}) virus or the double mutant variant (H9N2-UDL$^{PA\text{-}E349K\ PB2\text{-}M631L}$) containing the PA-E349K and PB2-M631L mutations. The inoculated eggs were incubated at 37.5°C. Allantoic fluids were collected at the indicated timepoints. Data are PFU/ml in allantoic fluids measured by plaque assay and statistically analysed by multiple unpaired two-sample T-test. Statistical annotations are defined as *ns=not significant*.

d, Human airway epithelial cells were infected with human-adapted H1N1 virus (A/England/195/2009) (England/195) or H9N2-UDL^{WT} virus or H9N2-UDL$^{PA\text{-}E349K\ PB2\text{-}M631L}$ and incubated at 37.0°C. Cell culture supernatants were harvested at the indicated timepoints and titrated by plaque assays. Data was statistically analysed by multiple unpaired two-sample T-test to compare growth of England/195 virus with growth of H9N2-UDL^{WT} virus or H9N2-UDL$^{PA\text{-}E349K\ PB2\text{-}M631L}$ at each timepoint. Data was analysed by one-way ANOVA and Dunnett's multiple comparison test. Statistical annotations are defined as **=P≤0.05, **=P≤0.01*.

e, WT embryonated eggs were inoculated with 100 PFU of a mixture of H9N2-UDL^{WT} and H9N2-UDL$^{PA\text{-}E349K\ PB2\text{-}M631L}$ virus containing <10% of the mutant virus. The inoculated eggs were incubated at 37.5°C. Allantoic fluids were collected at the indicated timepoints and followed by viral RNA purification. Next generation sequencing was performed on purified viral RNA to determine variant frequency in each egg.

f, Human airway epithelial cells were infected with a mixture of H9N2-UDL^{WT} virus and H9N2-UDL$^{PA\text{-}E349K\ PB2\text{-}M631L}$ virus containing <20% of the mutant virus and incubated at 37.0°C. Cell culture supernatants were harvested at the indicated timepoints and followed by viral RNA purification. Next generation sequencing was performed on purified viral RNA to determine variant frequency at each timepoint.

g-h, Location of amino acids mutated in virus isolated from ANP32A$^{N129I\text{-}D130N}$ chickens in the asymmetric polymerase dimer in combination with chANP32A (PDB:6XZP) **(g)** or the symmetric polymerase dimer (PDB:6QXB) were plotted using ChimeraX **(h)**. **g,** Influenza virus asymmetric polymerase dimer (PDB: 6XZP) showing ANP32A in light green, amino acids 129 and 130 highlighted in purple, PB2-627K in red, PB2-M631L in orange, PA-E349K in yellow, PA-Q556R in dark green and PA-T691I in blue. **h,** Influenza virus symmetric polymerase (PDB: 6QX8) showing PB2-627K in red, PB2-M631L in orange, PA-E349K in yellow, PA-Q556R in dark green and PA-T691I in blue.

807 **Fig. 5. Deletion of ANP32 A, B, and E eliminates viral polymerase activity and viral proliferation.**

808 **a**, ANP32A deletion strategy: two gRNAs were used to generate a 15-kb deletion in ANP32A in male
809 and female PGCs. Clonal lines of ANP32A-knockout (AKO) PGCs were isolated, propagated and
810 injected into iCaspase9 host embryos which were incubated to hatch. After hatching, the surrogate
811 hosts were raised to sexual maturity and directly mated. All offspring from eggs laid by the surrogate
812 hosts were biallelic for ANP32A deletion (**See Supplementary Fig. 14**).

813 **b-c,** WT or ANP32A$^{N129I-D130N}$ or AKO 11-day-old embryonated eggs were inoculated with wildtype
814 H9N2-UDL (H9N2-UDL^{WT}) virus or the double mutant variant (H9N2-UDL$^{PA-E349K\ PB2-M631L}$) containing
815 the PA-E349K and PB2-M631L mutations. The inoculated eggs were incubated at 37.5°C. Allantoic
816 fluids were collected 48 hours later and PFU/ml measured by plaque assay. DL – detection limit of
817 plaque assay (10 PFU/ml). Data were statistically analysed by unpaired two-tailed T-test of
818 transformed data (Y=(Log(Y)). Statistical annotations are defined as *ns=not significant, **=P≤0.01,*
819 *****=P≤0.0001*.

820 **d,** PGCs were edited to delete ANP32A, ANP32B, or ANP32E or combinations of the deletion (**See**
821 **Supplementary Fig. 17**). PGCs were subsequently differentiated into fibroblast –like cells and used to
822 assay polymerase activity and viral replication.

823 **e,** Wildtype (WT) H9N2-UDL polymerase or the mutant isoform harbouring PA-E349K and PB2-
824 M631L mutations was reconstituted together with NP by plasmid transfection into chicken PGC-
825 derived fibroblast-like cells. Polymerase activity was measured at 48 hours post-transfection by
826 detection of *Firefly* luciferase signal generated from a minireplicon normalized to a *Renilla* luciferase
827 transfection control. Data shown are Firefly activity normalised to Renilla plotted as mean ± SEM
828 derived from three (n=3) independent experiments, each consisting of three technical replicates.
829 Data was statistically analysed by one-way ANOVA, and Dunnett's multiple comparison test to
830 compare polymerase activity in wildtype cells with activity in other cell lines. Error bars are SEM. ns=
831 not significant, *=P≤0.05, ****=P≤0.0001. Statistical annotations are defined as *ns= not significant,*
832 **=P≤0.05, ****=P≤0.0001*. *N129I-D130N* cells refer to cells with the homozygous ANP32A$^{N129I-D130N}$
833 genotype.

834 **f-g,** PGC-derived fibroblast-like cells were infected with a recombinant virus harbouring the HA and
835 NA genes of the H1N1 PR8 virus, and the polymerase and other internal genes from the highly
836 pathogenic H5N1 avian influenza virus A/turkey/Turkey/2005 (Tky05) (**f**) or a highly pathogenic
837 H5N1 clade 2.3.4.4b virus (A/chicken/Scotland/054477/2021) (**g**). Cell culture supernatants were
838 harvested at the indicated timepoints and titrated by plaque assays. Data was statistically analysed
839 by one-way ANOVA and Dunnett's multiple comparison test to compare virus growth in wildtype
840 cells with virus growth in other cell types at each timepoint. Statistical annotations are defined as
841 *ns=not significant, *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001*.

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Inoculation route	Genotype	Bird #	Day	PB2	PB1	PA	NS1/NEP
Low Dose - Direct Inoculation	WT	505	D3	-	-	-	-
Low Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	5692	D3	S489P	-	T639I	-
Low Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	5692	D6	G74S (43%) S489P (fixed)	I517L (17%)	**E349K (20%)** T639I (75%)	L52M (34%)
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	941	D2	**M631L (65%)**	-	T639I (24%)	-
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	941	D3	**M631L (87%)**	-	**E349K (72%)**	-
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	946	D2	-	-	-	-
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	946	D3	-	-	-	-
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	946	D4	-	-	**E349K (87%)**	G168V / D11Y (87%)
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	947	D6	**M631L**	-	-	-
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	949	D2	-	-	Q556R (96%)	-
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	949	D3	**M631L**	-	Q556R (10%)	G45R
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	949	D4	**M631L (74%)**	-	Q556R (23%)	G45R (74%)
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	950	D2	-	-	**E349K (35%)**	-
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	950	D3	**M631L (15%)**	-	**E349K (70%)**	-
High Dose - Direct Inoculation	ANP32A$^{N129I-D130N}$	950	D4	I570L (30%) **M631L (36%)**	K578T (15%)	**E349K (64%)** S409I (64%)	G211E / D54N (35%)
High Dose - Naïve Contact	WT	923	D6	-	-	**E349K (poly)**	-
High Dose - Naïve Contact	WT	923	D7	**M631L**	-	L345F (poly)	-

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848 **Table 1. Amino acid substitutions at consensus level in viral isolates recovered from swabs of the**
849 **oropharyngeal cavity of ANP32A$^{N129I-D130N}$ chickens and single infected WT sentinel chicken.**

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